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Permalink

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Journal

Journal of breath research, 11(1)

ISSN

1752-7155

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Publication Date

2017-02-01

DOI

10.1088/1752-7163/aa5833

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Peer reviewed



PAPER

Stable isotope profiles reveal active production of VOCs from human-associated microbes

RECEIVED

21 December 2016

ACCEPTED FOR PUBLICATION

10 January 2017

PUBLISHED

6 February 2017

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Volatile organic compounds (VOCs) measured from exhaled breath have great promise for the diagnosis of bacterial infections. However, determining human or microbial origin of VOCs detected in breath remains a great challenge. For example, the microbial fermentation product 2,3-butanedione was recently found in the breath of Cystic Fibrosis (CF) patients; parallel culture-independent metagenomic sequencing of the same samples revealed that *Streptococcus* and *Rothia* spp. have the genetic capacity to produce 2,3-butanedione. To investigate whether the genetic capacity found in metagenomes translates to bacterial production of a VOC of interest such as 2,3-butanedione, we fed stable isotopes to three bacterial strains isolated from patients: two gram-positive bacteria, *Rothia mucilaginosa* and *Streptococcus salivarius*, and a dominant opportunistic gram-negative pathogen, *Pseudomonas aeruginosa*. Culture headspaces were collected and analyzed using a gas chromatographic system to quantify the abundance of VOCs of interest; mass spectroscopy was used to determine whether the stable isotope label had been incorporated. Our results show that *R. mucilaginosa* and *S. salivarius* consumed D-Glucose-¹³C₆ to produce labeled 2,3-butanedione. *R. mucilaginosa* and *S. salivarius* also produced labeled acetaldehyde and ethanol when grown with ²H₂O. Additionally, we find that *P. aeruginosa* growth and dimethyl sulfide production are increased when exposed to lactic acid in culture. These results highlight the importance VOCs produced by *P. aeruginosa*, *R. mucilaginosa*, and *S. salivarius* as nutrients and signals in microbial communities, and as potential biomarkers in a CF infection.

Introduction

Breath metabolites have incredible potential to serve as non-invasive diagnostics of bacterial infection and other diseases, but the origins of the metabolites are often unknown. Microbes contribute significantly to the complex mix of molecules present in the human body. An average human likely contains >200 000 metabolites, and half or more of them are produced by or altered by the microbes that reside in and on humans (Wikoff *et al* 2009, Dorrestein *et al* 2014, Sharon *et al* 2014). Furthermore, most of the genes that distinguish one person from another are encoded by the microbial community that inhabits each person; human-associated microbes encode at least an

order of magnitude more genes than the human genome (McFall-Ngai *et al* 2013). A very early attempt to profile breath and urine metabolites by Linus Pauling at Caltech in 1971 showed that starving the gut microbes led to more consistent breath and urine profiles across study participants (Pauling *et al* 1971). More recent studies have sampled breath VOCs to diagnose or detect bacterial infections in pneumonia (Fijten *et al* 2015) and tuberculosis (Phillips *et al* 2010).

Some of the metabolites produced by human-associated microbes are volatile, have the capacity to travel and affect the physiology of neighboring microbial and human cells, and can be captured by breath sampling (Létoffé *et al* 2014, Audrain *et al* 2015). For example, 2,3-butanedione was detected in the breath

Table 1. Ten top microbes in the CF airway and some of the VOCs they produce.

Microbe	VOCs	Supporting literature
<i>Pseudomonas aeruginosa</i>	2-butanone, 2-aminoacetophenone, dimethylsulfide, and hydrogen cyanide	Preti <i>et al</i> 2009, Scott-Thomas <i>et al</i> 2010, Smith <i>et al</i> 2013,
<i>Streptococcus pneumoniae</i>	Benzaldehyde, benzylalcohol, acetic acid, and acetaldehyde	Preti <i>et al</i> 2009, Filipiak <i>et al</i> 2012
<i>Staphylococcus aureus</i>	Isovaleric acid, 2-methylbutyric acid, and isobutyric acid	Preti <i>et al</i> 2009
<i>Rothia mucilaginosa</i>	Acetaldehyde	Moritani <i>et al</i> 2015
<i>Veillonella</i>	Propionic acid, acetic acid, and isovaleric acid	Dumont <i>et al</i> 1978
<i>Prevotella</i>	Acetic and succinic acids	Downes <i>et al</i> 2007
<i>Haemophilus influenza</i>	Indole, benzaldehyde, acetic acid, and benzylalcohol	Preti <i>et al</i> 2009, Filipiak <i>et al</i> 2012
<i>Stenotrophomonas maltophilia</i>	Methylpyrazine, dimethylpyrazine, acetophenone, and caprolactam	Preti <i>et al</i> 2009
<i>Burkholderia</i> spp.	Acetic acid, dimethyl disulfide, and methylcyclohexane	Tenorio-Salgado <i>et al</i> 2013
<i>Granulicatella</i>	N/A	N/A

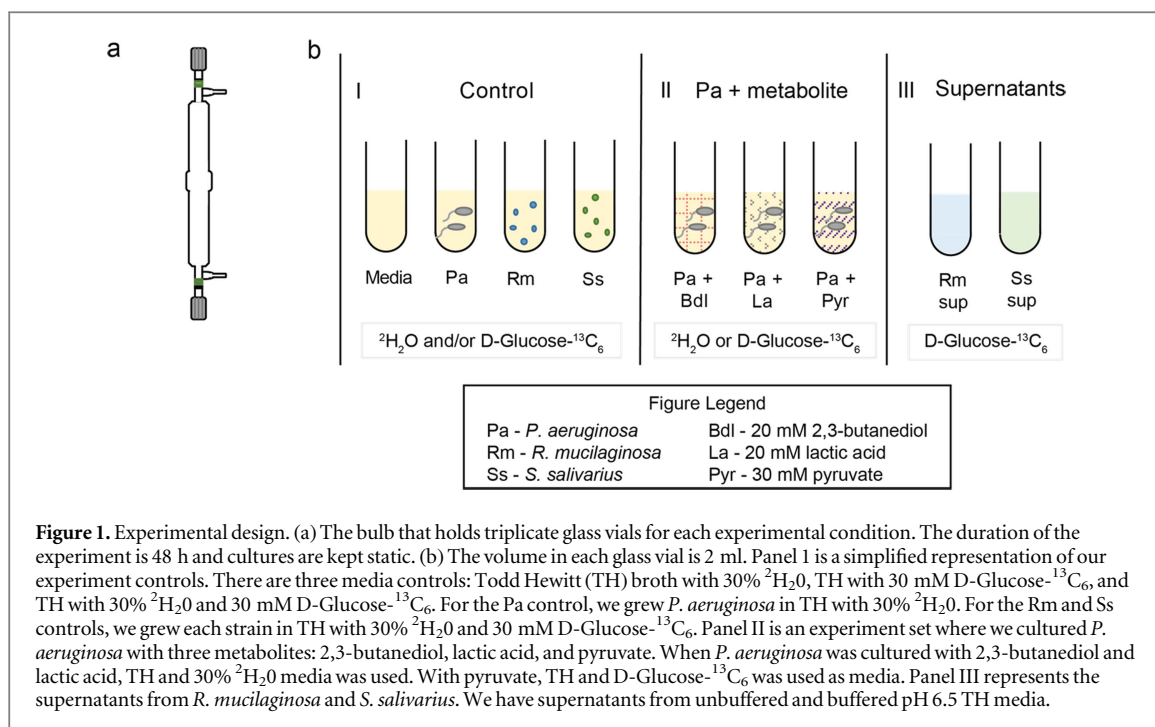
of Cystic Fibrosis (CF) patients (Whiteson *et al* 2014). One challenge in identifying the cellular origin of volatile organic compounds (VOCs) is determining whether the metabolites are produced by microbial or human cells. Humans and bacteria share several fundamental pathways and have the ability to produce many of the same volatile molecules, such as isoprene and acetone, the two most abundant VOCs detected in breath (Fenske and Paulson 1999, Miekisch *et al* 2004, Thorn and Greenman 2012). In the case of 2,3-butanedione, a subset of microbes encode the enzymes from this fermentation pathway, however 2,3-butanedione is not known to be a product of human metabolism. Parallel metagenomic sequencing from CF patients with 2,3-butanedione in their breath showed *Streptococcus* and *Rothia* spp. contain the acetoin pathway genes leading to the production of 2,3-butanedione (Whiteson *et al* 2014). A similar companion study also produced in this special issue of the Journal of Breath Research shows the taxonomy of microbial genes responsible for the production of acetaldehyde and ethanol (Bos *et al* 2016). To determine whether 2,3-butanedione and other volatile molecules of interest such as acetaldehyde and ethanol are actively produced by bacterial isolates from CF patients, we employed the use of labeled stable isotopes fed to *in vitro* cultures to track bacterial metabolism.

This study focuses on several bacterial isolates from CF patients that are often found in the airways when the natural lung clearance mechanisms are impaired. Therefore, the molecules that are produced by these bacteria are potentially relevant for CF, chronic obstructive pulmonary disorder, and pneumonia. CF patients' lungs are chronically infected with opportunistic pathogens that significantly alter the health and physiology of the patient. Different types of infections can lead to varying patient outcomes (Hauser *et al* 2011, Ahlgren *et al* 2015). Among the most dominant bacteria are *Pseudomonas aeruginosa*, *Burkholderia* species, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae* (table 1). *Rothia mucilaginosa* has also been found to be a dominant strain in culture-independent studies

of CF airway microbial communities (Lim *et al* 2013), and is highly abundant in CF sputum samples (Blainey *et al* 2012, Fodor *et al* 2012, Paganin *et al* 2015).

Our goal was to track microbial production of VOCs in clinically relevant CF bacteria. Specifically, we were interested in *P. aeruginosa*, *R. mucilaginosa*, and *Streptococcus salivarius*. *P. aeruginosa* is a widespread opportunistic pathogen in CF which becomes more dominant through time in decades-long chronic CF infections. The recent detection of 2,3-butanedione in the breath of CF patients, along with the genes necessary for acetoin metabolism from metagenomic sequencing of the same samples (Whiteson *et al* 2014), motivated us to see if we could verify that CF isolates are indeed producing 2,3-butanedione. Analysis of culture-independent metagenomic sequence reads from CF sputum showed that *R. mucilaginosa* and a variety of *Streptococcus* spp. encode genes involved in the production of 2,3-butanedione (Whiteson *et al* 2014), which induces virulence in *P. aeruginosa* (Venkataraman *et al* 2011, 2014, Whiteson *et al* 2014) and has also been found to be present in the breath of CF patients (Whiteson *et al* 2014). *R. mucilaginosa* is an abundant and understudied bacteria common in all human oral-microbial communities, including CF sputum, and it is known to produce the toxic volatile acetaldehyde (table 1) (Moritani *et al* 2015). *Streptococcus* spp. are also common in all human oral microbial communities. A large fraction of the metagenomic reads that map to the 2,3-butanedione pathway—25% to 85%—originated from *Streptococcus* (Whiteson *et al* 2014). The sequences from CF sputum mapped equally well to several strains of *Streptococcus* spp., including *S. parasanguinus*, *S. salivarius* and *S. pneumoniae*. We included a strain of *S. salivarius* cultured from a CF patient which is capable of producing 2,3-butanedione based on genome sequence data.

To track VOCs produced by microbial metabolism, we used stable isotopes (D-Glucose-¹³C₆ and ²H₂O), sampled headspaces of *in vitro* cultures, and analyzed these headspace samples using a gas chromatography multi column/detector system. Although metagenomic evidence suggests that *Streptococcus* spp.



and *R. mucilaginosa* may produce 2,3-butanedione (Whiteson *et al* 2014), this study used stable isotopes to confirm its production by CF isolates. We also set up cultures of a clinical isolate of *P. aeruginosa* to study VOC production with and without 2,3-butanediol and other physiologically relevant products of *S. salivarius* and *R. mucilaginosa*. Future investigation for optimal *P. aeruginosa* growth conditions in supernatants from other bacteria is one of our goals in understanding how *P. aeruginosa* is affected by the neighboring CF community.

The airways of people with CF have a lower pH than healthy airways, and immediately prior to a pulmonary exacerbation event, the pH drops even lower (Tate *et al* 2002, Ojoo *et al* 2005, Pezzulo *et al* 2012, Quinn *et al* 2015). To investigate the effect of low pH on our clinical isolates, our experimental conditions also involved the use of lactic acid or buffer to adjust the media pH. In summary, results show that levels of 2,3-butanedione, acetaldehyde, and dimethyl sulfide are increased when the pH is decreased and these signatures can be used to distinguish the metabolisms of *P. aeruginosa*, *R. mucilaginosa*, and *S. salivarius*.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains chosen for this study were isolated from CF patients at the UCSD Adult CF Clinic: *Pseudomonas aeruginosa* PaFLR01, *Rothia mucilaginosa* RmFLR01, and *Streptococcus salivarius* SsFLR02. Strains were grown at 37 °C for 24 h in Todd Hewitt (TH) broth (BD Bacto, Fisher Scientific). From the 24 h cultures, triplicate OD₅₀₀ 0.05

cultures were started in TH broth with 30 mM D-Glucose- $^{13}\text{C}_6$ (Sigma-Aldrich and Cambridge Isotope Laboratory), unless otherwise noted. Some conditions involve the addition of 30% $^2\text{H}_2\text{O}$ (Sigma-Aldrich) or 100 mM pH 6.5 potassium phosphate buffer (Fisher Scientific). We used OD₅₀₀ to measure cell densities, instead of OD₆₀₀ that is traditionally used, because *P. aeruginosa* strain PaFLR01 produces pyocyanin, a colorful phenazine that absorbs at 691 nm.

P. aeruginosa pyocyanin measurement

At the end of each experiment, *P. aeruginosa* cultures were centrifuged to pellet cells and supernatants were collected to read absorbance measurements at 691 nm with a spectrophotometer.

Headspace gas capture and gas chromatography

A modified bioreactor and a gas chromatographic (GC) system were used for headspace gas collection and VOC analysis (Colman *et al* 2001, Umber *et al* 2013). In particular, replicates of three individual glass culture tubes were placed in the bulb of the bioreactor (figure 1(a)) and, in order to replace any laboratory air, the reactor was flushed for 6 min at 1.5 l min⁻¹ with ultra clean air collected at 10 000 feet at the Crooked Creek Research station in White Mountain (California). After flushing, the bioreactor was incubated at 37 °C for 48 h statically and the headspace was collected and analyzed using a GC system as described in previous works (Colman *et al* 2001, Shin *et al* 2009, Umber *et al* 2013, Whiteson *et al* 2014). Briefly, headspace sample was cryogenically pre-concentrated in a stainless steel loop filled with glass beads. The sample was re-vaporized using

hot water (at approximately 80 °C) and split into five streams directed to a multi-column/detector GC system (all Hewlett Packard 6890) using UHP helium as carrier gas. The system consists of three Hewlett-Packard 6890 GC units. The first GC is equipped with two different column combinations output to an electron capture detector (ECD) and an FID; the second GC is output to an FID; and the third GC is equipped with two different column combinations and output to a quadrupole mass spectrometer detector working in selected ion monitoring (SIM) mode, and an ECD. The MSD is set to operate in SIM mode and scan mode simultaneously.

The different VOCs are expressed in part per trillion by volume. The average from the triplicate samples and a one-tailed student's t-test was used to identify VOCs that were significantly elevated compared to the media control.

Genome annotation

P. aeruginosa and *S. salivarius* genomes were sequenced on the Illumina MiSeq platform at San Diego State University. *R. mucilaginosa* was sequenced on the Illumina MiSeq platform at microbesNG at the University of Birmingham. All sequenced genomes were uploaded onto RAST for annotation (Aziz et al 2008).

Experimental design

Figure 1 displays the conditions of the experiment. All conditions were tested as biological triplicates in 2 ml volumes for 48 h, static. The media control included three conditions: TH broth with $^2\text{H}_2\text{O}$, TH broth with D-Glucose- $^{13}\text{C}_6$, and TH with both $^2\text{H}_2\text{O}$ and D-Glucose- $^{13}\text{C}_6$. The *P. aeruginosa* control included two conditions: *P. aeruginosa* grown in TH with $^2\text{H}_2\text{O}$ or TH with D-Glucose- $^{13}\text{C}_6$ and pyruvate. The *R. mucilaginosa* and *S. salivarius* controls included each strain grown in TH with $^2\text{H}_2\text{O}$ and D-Glucose- $^{13}\text{C}_6$ (Panel I). *P. aeruginosa* was cultured in TH with the addition three metabolites: 20 mM 2,3-butanedione, 20 mM lactic acid, and 30 mM pyruvate (Panel II). We collected *R. mucilaginosa* and *S. salivarius* supernatants to perform cross-feeding experiments with *P. aeruginosa*, but *P. aeruginosa* did not grow. Therefore, we excluded that portion of the data set, but still kept the supernatants (Panel III) because the strains were cultured with a single labeled isotope, D-Glucose- $^{13}\text{C}_6$.

CFC-11 is consistent across all samples

Trichlorofluoromethane (CFC-11) is a VOC we do not expect to see produced by our strains and is constant across all samples (supplementary figure 2). Therefore, CFC-11 is a good negative control for the GC system.

Results and discussion

Production of 2,3-butanedione, acetaldehyde, and ethanol

2,3-butanedione, acetaldehyde, and ethanol are fermentation products derived from pyruvate metabolism (figure 2). As part of glycolysis, glucose is converted to pyruvate. Following pyruvate metabolism, pyruvate can either be converted to acetolactate or acetaldehyde by the enzymes acetolactate synthase (BudB, E.C. 2.2.1.6) or pyruvate decarboxylase (Pdc, E.C. 4.1.1.1), respectively (figure 2). Acetolactate is then broken down to 2,3-butanedione and acetoin by the enzyme BudA (E.C. 4.1.1.5; figure 2). Acetaldehyde can be converted to ethanol by alcohol dehydrogenase (ADH, E.C. 1.1.1.1; figure 2); the reaction is reversible. By using D-Glucose- $^{13}\text{C}_6$, we expect to see labeled VOCs derived from pyruvate metabolism, specifically 2,3-butanedione, acetaldehyde, and ethanol. We expect *R. mucilaginosa* and *S. salivarius* to consume glucose, but glucose is not a preferred carbon source for *P. aeruginosa*, so we included $^2\text{H}_2\text{O}$ in our experiments in order to track active *P. aeruginosa* metabolism.

When *R. mucilaginosa* and *S. salivarius* consume D-Glucose- $^{13}\text{C}_6$, our data show that ^{13}C was incorporated into 2,3-butanedione. We attribute labeled 2,3-butanedione to *R. mucilaginosa* and *S. salivarius* metabolism because while there was unlabeled 2,3-butanedione in the media and *P. aeruginosa* controls, ^{13}C was only incorporated when D-Glucose- $^{13}\text{C}_6$ was metabolized by *R. mucilaginosa*, and *S. salivarius* (figure 3(a)). Genetic evidence also supports these findings. *P. aeruginosa*, *R. mucilaginosa*, and *S. salivarius* have the genetic capability to convert pyruvate to acetolactate by BudB (E.C. 2.2.1.6) (figure 2). Only *R. mucilaginosa* and *S. salivarius* have the genetic capacity to convert acetolactate to 2,3-butanedione and acetoin through BudA (E.C. 4.1.1.5) (figure 2).

Through fermentation, pyruvate can be converted to acetaldehyde by pyruvate decarboxylase (Pdc, E.C. 4.1.1.1; figure 2). An example of an *in vitro* study of milk fermentation demonstrated through use of labeled isotopes that *Streptococcus thermophilus* was able to convert ^{13}C -glucose to acetaldehyde (Ott et al 2000). Surprisingly, while the CF isolates of *R. mucilaginosa* and *S. salivarius* produced acetaldehyde and ethanol, the cultures grown with D-Glucose- $^{13}\text{C}_6$ did not lead to production of ^{13}C -labeled acetaldehyde or ethanol (shaded areas of figures 3(b) and (c)). When *R. mucilaginosa* and *S. salivarius* are cultured in media with $^2\text{H}_2\text{O}$, both acetaldehyde and ethanol are labeled (unshaded areas of figures 3(b) and (c)). *P. aeruginosa* cultures did not produce any of the fermentation products shown in figure 3 in levels higher than the media controls. Labeled acetaldehyde was also detected in the media control when $^2\text{H}_2\text{O}$ was present. We expected deuterium incorporation into acetaldehyde to depend on bacterial enzymes. However, acetaldehyde is a weak

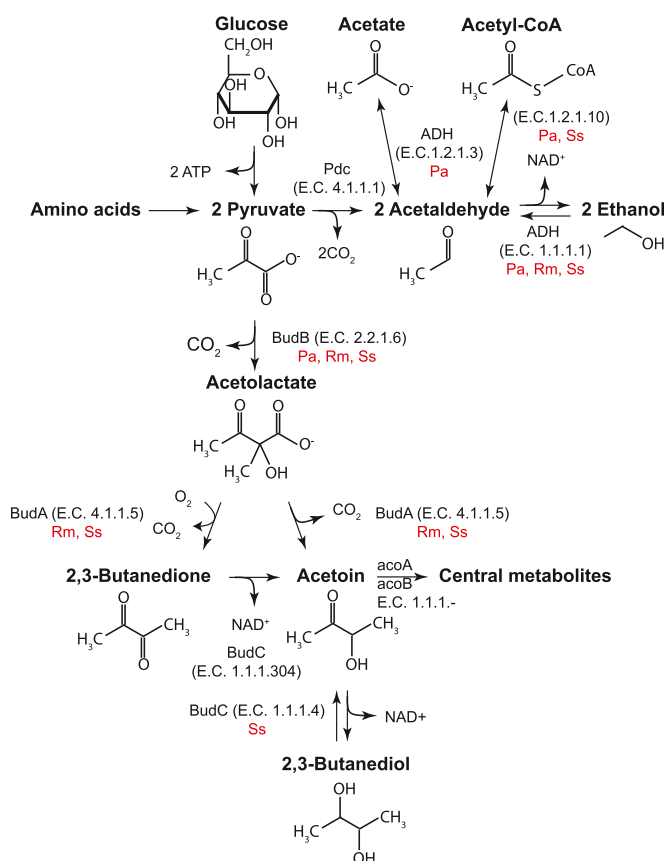


Figure 2. A simplified schematic of pyruvate metabolism. In red are the strains that have the listed genes in their genome.

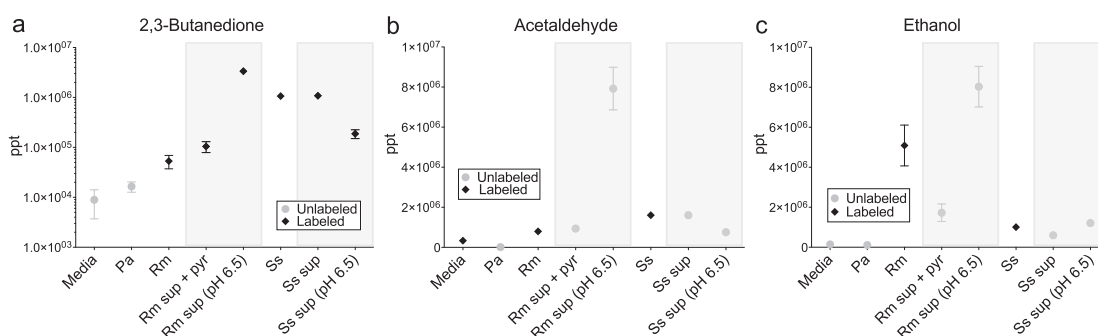


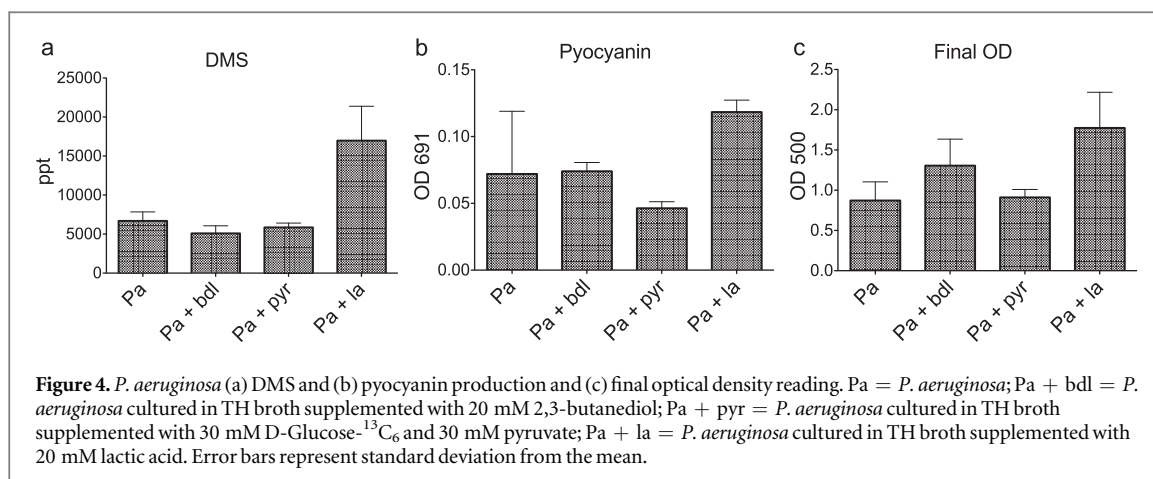
Figure 3. 2,3-Butanedione, acetaldehyde, and ethanol production. (a) 2,3-butanedione, (b) acetaldehyde, and (c) ethanol unlabeled and labeled concentrations in ppt. Media = average values of three media controls; Pa = *P. aeruginosa*; Rm = *Rothia mucilaginosa*; Rm sup = spent medium from *Rothia mucilaginosa* after 48 h of growth in TH broth supplemented with D-Glucose- $^{13}\text{C}_6$; Ss = *S. salivarius*; Ss sup = spent medium from *Streptococcus salivarius* after 24 h of growth in TH broth supplemented with D-Glucose- $^{13}\text{C}_6$. Error bars represent standard deviation from the mean. Shaded box represents conditions where only a single labeled isotope was used —D-Glucose- $^{13}\text{C}_6$. Unshaded areas display samples that were grown in the presence of 30% $^2\text{H}_2\text{O}$ and D-Glucose- $^{13}\text{C}_6$.

acid, and in the presence of base in the media the deuterium exchange with the alpha carbons of acetaldehyde could occur.

Acetaldehyde production may rely on acetate, acetyl-CoA, pyruvate, L-threonine, ethanol, or 2-deoxyribose-5-phosphate (Ott *et al* 2000). Our results suggest that acetaldehyde and ethanol production bypass glucose. Although both *R. mucilaginosa* and *S. salivarius* have the capacity to convert glucose to pyruvate, RAST annotations of the two strains suggest they do not have the genetic capability to convert

pyruvate to acetaldehyde by pyruvate decarboxylase (E.C. 4.1.1.1; figure 2). Because the acetaldehyde and ethanol we detected in *R. mucilaginosa* and *S. salivarius* cultures are labeled in the presence of $^2\text{H}_2\text{O}$ but not D-Glucose- $^{13}\text{C}_6$, they may share a common pathway that involves the hydrolysis of $^2\text{H}_2\text{O}$.

Based on genome annotations (figure 2), *P. aeruginosa*, *R. mucilaginosa*, and *S. salivarius* are genetically capable of converting ethanol to acetaldehyde by alcohol dehydrogenase (E.C. 1.1.1.1) (figure 2). *S. salivarius* is also genetically capable of converting acetyl-CoA



to acetaldehyde by acetaldehyde dehydrogenase (E.C. 1.2.1.10) (figure 2). *P. aeruginosa* has the genetic capability to convert acetate or acetyl-CoA to acetaldehyde through enzymes aldehyde dehydrogenase (E.C. 1.2.1.3) and acetaldehyde dehydrogenase (E.C. 1.2.1.10), respectively (figure 2). However, the culturing conditions did not lead *P. aeruginosa* to produce significant levels of acetaldehyde compared to *R. mucilaginosa* or *S. salivarius*, and acetaldehyde and ethanol were not labeled in the presence of ²H₂O in the *P. aeruginosa* cultures.

Decreasing the pH of TH broth to pH 6.5 by adding 100 mM potassium phosphate (figure 3) led to an increase in growth and an even greater increase in the production of ¹³C labeled 2,3-butanedione and unlabeled acetaldehyde by *R. mucilaginosa* (figures 3(a) and (b); OD₆₀₀ of unbuffered cultures reached 0.73–0.76 while the OD₆₀₀ of the pH 6.5 culture reached 2.48–2.55). *S. salivarius*, on the other hand, showed higher 2,3-butanedione production in unbuffered media compared to buffered pH 6.5 media. To verify the presence and measure the concentrations of 2,3-butanedione in our samples, Voges–Proskauer assays were performed on supernatants (supplementary figure 1). The results were consistent with our gas chromatography findings, in that we detected high levels of 2,3-butanedione in the *R. mucilaginosa* supernatant (figure 3(a) and supplementary figure 1).

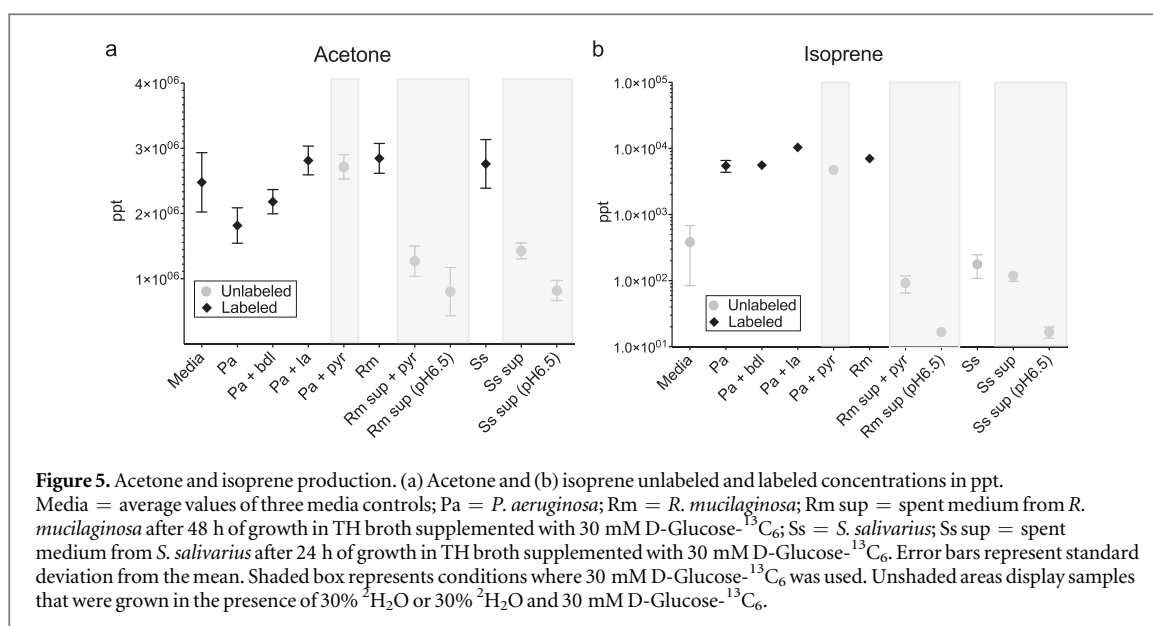
Some limitations of studying pH dependent microbial physiology include the additional nutrients provided by the buffer (i.e., perhaps the phosphate buffer provides phosphate which boosts growth); buffered media may also eradicate micro-gradients relevant in the CF lung. For example, *R. mucilaginosa* produced more 2,3-butanedione in buffered pH 6.5 media while *S. salivarius* produced more when grown in unbuffered media. *S. salivarius* cultured in unbuffered media may include micro-gradients that are even more acidic than the buffered pH 6.5 media. Early dairy literature demonstrated that as pH becomes more acidic, *Streptococci* produce a higher yield of 2,3-butanedione (diacetyl) (Michaelian et al 1938). Without the restriction of a buffered environment, *S.*

salivarius has the ability to further decrease the pH of its micro-environment. Therefore, the local pH may be much lower in an unbuffered condition than the buffered pH 6.5 media, driving the physiology of a subpopulation of cells. While buffers are useful to control the pH, especially for bulk measurements of VOCs like those presented here, future studies using pH sensitive dyes and imaging may allow observation of the local spatial structure of interactions.

***P. aeruginosa* physiology is affected by exposure to microbial metabolites**

P. aeruginosa is a dominant opportunistic pathogen in CF and for this reason, to understand the polymicrobial community dynamics, it is essential to investigate its role and interactions with other bacteria and their metabolites. When *P. aeruginosa* was exposed to 2,3-butanediol, lactic acid, and pyruvate in TH broth, not only did we detect production of dimethyl sulfide (DMS; figure 4(a)), we also detected production of pyocyanin in the culture (figure 4(b)). DMS is a VOC that is well studied in the marine food web and is important in environmental sciences (Yoch 2002, Carrión et al 2015); DMS has also been detected in the breath of CF patients (Kamboures et al 2005), and *P. aeruginosa* has been shown to produce DMS and stimulate the growth of *Aspergillus* (Briard et al 2016). The stable isotope labeled substrates (²H₂O and D-Glucose-¹³C₆) did not lead to label incorporation into DMS, potentially because glucose is not a preferred carbon source for *P. aeruginosa*, and DMS production may not depend on ²H₂O exchange.

Pyocyanin is an active redox phenazine produced by *P. aeruginosa* and found in the lungs of CF patients (Mentel et al 2009, Hunter et al 2012, Glasser et al 2014). Interestingly, DMS, pyocyanin production, and growth are higher when *P. aeruginosa* is cultured with 20 mM lactic acid (figures 4(a) and (b)). Lactic acid both lowers pH and is a preferred carbon source for *P. aeruginosa*, which could lead to better growth and therefore increased production of DMS, pyocyanin and other metabolic products. In our conditions, neither *R. mucilaginosa* nor *S. salivarius* produce DMS.



As such, we find that among our strains, DMS production is unique to *P. aeruginosa*.

Production of acetone and isoprene

Acetone and isoprene are the two most abundant VOCs detected in breath and can be produced by either human or bacterial cells. In our study, we find that acetone was detected as a labeled volatile in each of the media controls and experimental conditions where ²H₂O was present (figure 5(a)). Therefore, the production of acetone cannot be attributed to any of the strains because of its presence in the media controls. The use of ²H₂O is not a reliable method for identifying metabolic production of acetone and potentially other VOCs including butanal, 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, and 2-butanone (data not shown) because the deuterium from the heavy water may be exchanging with hydrogens in the media.

Isoprene levels were higher in both *P. aeruginosa* and *R. mucilaginosa* cultures, and it was labeled when *P. aeruginosa* and *R. mucilaginosa* were cultured in media with ²H₂O (figure 5(b)). Labeled isoprene was not detected in the media controls or in *P. aeruginosa* cultures lacking ²H₂O, suggesting that label incorporation was due to bacterial metabolism. It is not clear why isoprene levels were sometimes lower in *R. mucilaginosa* and *S. salivarius* cultures. *P. aeruginosa*, *R. mucilaginosa*, and *S. salivarius* share genes in the isoprenoid biosynthesis pathway, farnesyl diphosphate synthase (E.C. 2.5.1.10) and geranylgeranyl pyrophosphate synthetase (E.C. 2.5.1.29). *P. aeruginosa* also has several other genes involved in the isoprenoid pathway, but neither *P. aeruginosa* nor *R. mucilaginosa* have isoprene synthase (IspS, E.C. 4.2.3.27), the gene required to convert dimethylallyl diphosphate (DMAPP) to isoprene. There may be other mechanisms or pathways that allow *P. aeruginosa* and *R. mucilaginosa* to produce isoprene. Other bacteria that have

been shown to produce isoprene are *Bacillus subtilis* (Julsing et al 2007, Xue and Ahning 2011) and *Escherichia coli* (Zhao et al 2011, Zurbriggen et al 2012). Although isoprene was detected as labeled in *R. mucilaginosa* control cultures, it was not detected in the supernatants. Further investigation is needed to understand the role of microbial metabolism in isoprene production.

Conclusions

Analysis of VOCs in breath often leads to the detection of molecules that could be of human or microbial origin. Our microbial culture experiments using stable isotope labeled substrates demonstrate that microbial metabolism is responsible for the production of several VOCs found in breath, including 2,3-butanedione, acetaldehyde, ethanol, and isoprene. GC analysis and Voges–Proskauer test results have confirmed that clinical isolates *R. mucilaginosa* and *S. salivarius* produce 2,3-butanedione, which has previously been supported by metagenomics data (Whiteson et al 2014). Acetaldehyde is an important molecule for human health because it is a toxic carcinogenic molecule that is produced by members of the oral microbial community. Although it can be derived from pyruvate, acetaldehyde production can start from other substrates, including acetyl-CoA, acetate, 2-deoxyribose-5-phosphate, ethanol, and L-threonine (Ott et al 2000). We found that our *P. aeruginosa* strain produced DMS, which is more abundant when *P. aeruginosa* is cultured in TH supplemented with lactic acid. Finally, we found that labeled isoprene was produced by *P. aeruginosa* and *R. mucilaginosa*. Isoprene is one of the most abundant VOCs detected in breath, but when compared to other studies that measure concentrations of isoprene, our strains produced much less than what has been detected in breath

(King *et al* 2009, Khan *et al* 2013). This approach is promising for teasing apart the interactions that affect breath VOC production in human-associated microbial communities. For example, future studies that involve feeding stable isotope labeled substrates to one type of bacteria, and cross-feeding the metabolic products to another bacteria, could help us understand metabolic interdependencies and their VOC signatures in polymicrobial infections. As non-invasive diagnoses are being developed, identifying the contributing bacteria of an infection in CF is ideal for employing more specific and timely treatments. Lab results may take several days while results from breath samples can be instantaneous. Studies focused on the microbial origin of volatile molecules are critical for using breath data for earlier diagnosis and targeted treatments of infections.

Acknowledgments

We are grateful to members of the Whiteson and Blake groups, Dr Lieuwe D J Bos, and Dr Heather Maughan for comments on the manuscript. This independent research was supported by the Gilead Sciences Research Scholars Program in Cystic Fibrosis. Our isolates and initial momentum came from Dr Doug Conrad, Director of the UCSD Adult CF Clinic, Dr Robert Quinn, Mike Furlan, Dr Yan Wei Lim, and Prof Forest Rohwer during the era of the CF Expedition team, and we are grateful for years of fun conversation and ideas.

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